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THE TOXICITY OF MENADIONE AND MITOZANTRONE IN HUMAN LIVER-DERIVED HEP G2 HEPATOMA CELLS

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Abstract—The cytotoxic properties of quinone drugs such as menadione and adriamycin® are thought to be mediated through one-electron reduction to semiquinone free radicals. Redox cycling of the semiquinones results in the generation of reactive oxygen species and in oxidative damage. In this study the toxicity of mitozantrone, a novel quinone anticancer drug, was compared with that of menadione in human Hep G2 hepatoma cells. Mitozantrone toxicity in these cells was not mediated by the one-electron reduction pathway. In support of this, inhibition of the enzymes glutathione reductase and catalase, responsible for protecting the cells from oxidative damage, did not affect the response of the Hep G2 cells to mitozantrone, whereas it exacerbated menadione toxicity. In addition, the toxicity of menadione was preceded by depletion of reduced glutathione which was probably due to oxidation of the glutathione. Mitozantrone did not cause glutathione depletion prior to cell death. DT-diaphorase activity and intracellular glutathione were found to protect the cells from the toxicity of both quinones. Inhibition of epoxide hydrolase potentiated mitozantrone toxicity but did not affect that of menadione. Our experiments indicate that mitozantrone toxicity may involve activation to an epoxide intermediate. Both quinone drugs inhibited cytochrome P-450-dependent mixed-function oxidase activity, although menadione was more potent in this respect.

Quinones occur widely in nature and have been extensively studied for their cytotoxic and antitumour properties. The quinone-containing anthracyclines adriamycin® (doxorubicin hydrochloride)† and daunorubicin are among the most widely prescribed drugs in the treatment of a wide range of human malignancies [1]. However, their use is severely limited by dose-dependent cardiotoxicity [2] and this problem has lead to the development of new, structurally-related anthraquinone drugs, such as mitozantrone. The toxic side effects, particularly cardiotoxicity, are much less severe with mitozantrone than with adriamycin® [3]. However, this compound does not exhibit the same broad spectrum of antitumour activity as does adriamycin, and it is currently used in the treatment of breast cancer [4]. Little is known about the metabolism and toxicity of mitozantrone in human tissues and in this study we have compared the cytotoxicity of mitozantrone with that of the model quinone, menadione (2-methyl-1,4-naphthoquinone), in the human liver-derived hepatoma cell line, Hep G2.

Quinones can undergo either one-electron reduction to yield the semiquinone free radical or two-electron reduction directly to the hydroquinone [5, 6]. The cytotoxic and antitumour properties of quinone drugs are thought to be mediated through the one-electron reduction to semiquinone free radicals [7, 8]. Most semiquinones are readily re-oxidised in aerobic conditions and can enter redox cycles with molecular oxygen, forming various reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen [6]. Formation of oxygen intermediates and the redox cycling of the quinone may ultimately lead to conditions of oxidative stress and cell toxicity. For instance, exposure of isolated rat hepatocytes to menadione results in production of superoxide, oxidation of glutathione and loss of cell viability [9]. The two-electron reduction pathway for quinones is considered relatively non-toxic and generates stable hydroquinones which can be readily conjugated.

The one-electron reduction of quinones is mainly catalysed by NADPH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase, and the flavoprotein NAD(P)H: (quinone acceptor) oxidoreductase, also known as DT-diaphorase, catalyses the two-electron reduction reaction [5, 6]. The activities of the enzymes glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase protect the cells from the effects of reactive oxygen species generated during the one-electron reduction of quinones [10]. There is also some evidence to suggest that mitozantrone may be metabolised by cytochrome P-450-dependent mixed-function oxidases (MFO)‡ in rat liver microsomes [11]. The involvement of one-electron and two-electron reduction, MFO and glutathione in the activation of mitozantrone and menadione to reactive intermediates and in their detoxification was investigated using specific enzyme

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† Adriamycin is a registered trademark of Farmitalia Carlo Erba.

‡ Abbreviations: GSH, reduced glutathione; GST, glutathione-*S*-transferase; EH, epoxide hydrolase; MFO, cytochrome P-450-dependent mixed-function oxidase; DEM, diethylmaleate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea, TCPO, 1,2-epoxy-3,3,3-trichloropropane; CDNB, 1-chloro-2,4-dinitrobenzene.

inhibitors and depletion of reduced glutathione (GSH). In addition, the ability of the two quinone drugs to inhibit drug metabolising enzymes was assessed using subcellular fractions of human adult liver.

MATERIALS AND METHODS

NADH, NADPH, glutathione reductase, cytochrome *c*, reduced and oxidised glutathione, dicoumarol, 1,2-epoxy-3,3,3-trichloropropane and 1,2-aminotriazole were obtained from Sigma (St Louis, MO). Foetal calf serum was from Gibco and Dulbecco's modification of Eagle's medium was from Flow Laboratories (Irvine, Scotland, U.K.) Phenanthrene - 9,10 - oxide, phenanthrene - 9,10 - dihydrodiol, ethoxyresorufin and resorufin were synthesised as described previously [12, 13]. Mitozantrone was a gift from Lederle Laboratories and 1,3-bis(2-chloroethyl)-1-nitrosourea was a gift from Bristol-Meyers. Hep G2 cells were obtained by Dr. W. T. Melvin, Department of Biochemistry, Aberdeen University, from Professor C. N. Hales, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

Culture and incubation of cells for toxicity studies. Hep G2 cells were routinely grown in monolayer or multilayer culture in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum. They were grown in a humidified atmosphere of 5% CO_2 in air and subcultured every 7 days at a split ratio of 1:3 (seeding density 3.5×10^6 cells on 75 cm^2 flasks). All enzyme activity measurements and toxicity studies were carried out on confluent cells between 7 and 10 days after subculture. Cell suspensions were prepared by treating confluent cultures with a 1:5 solution of 0.25% (w/v) trypsin: 0.02% (w/v) versene in phosphate buffered saline, pH 7.4. The viability of the suspensions was assessed by Trypan Blue exclusion (typically >90%). For toxicity experiments cells (10^6/ml) were incubated in 50 ml round-bottomed flasks in Krebs–Henseleit buffer, pH 7.4, containing 10 mM HEPES at 37° under an atmosphere of 95% O_2: 5% CO_2.

Cell suspensions were treated with either menadione (10–200 μM) or mitozantrone (50–200 μM) for up to 4 hr and samples removed at timed intervals for determination of cell viability (Trypan Blue exclusion) and GSH content [14]. The effect of various enzyme inhibitors and of prior GSH depletion on the toxicity of the two quinone drugs was investigated. DT-diaphorase activity was inhibited by adding 30 μM dicoumarol [6] to the cell suspensions at the same time as the drug. Cell suspensions were pretreated for 10 min with 1,2-aminotriazole to inhibit catalase activity [15]. Two millimoles 1,2-epoxy-3,3,3-trichloropropane (TCPO) was added directly to incubations as an inhibitor of microsomal epoxide hydrolase (EH) [16].

1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU) is a relatively specific inhibitor of glutathione reductase [17]. However, this agent depletes intracellular GSH in addition to inhibiting glutathione reductase. Eklow and coworkers developed a BCNU-treated isolated rat hepatocyte system in which glutathione reductase was inhibited, but GSH levels were normal [18] and this protocol has been modified for use with Hep G2 cells as follows. Hep G2 cells were exposed to 75 μM BCNU for 2 hr in monolayer culture in serum free medium. The cultures were then washed and incubated in medium containing 10% (v/v) foetal calf serum supplemented with 0.25 mM L-cysteine and GSH resynthesis allowed to proceed for a further 2 hr. Cell suspensions were then prepared and exposed to either menadione or mitozantrone. BCNU treatment did not affect the initial viability of the cell suspensions [control cells 93.2 ± 1.7% (N = 4) and BCNU-treated cells 92.0 ± 1.3% (N = 4) viable]. After BCNU treatment the activity of glutathione reductase was 37.0 ± 2.6 μmol/min/10^6 cells (N = 4) compared with 75.6 ± 3.2 μmol/min/10^6 cells (N = 4) in control cells. GSH content was 9.0 ± 1.9 nmol/10^6 cells (N = 3) in BCNU-treated cells compared with 8.0 ± 1.9 nmol/10^6 cells (N = 3) in the controls.

To deplete intracellular GSH, cell suspensions (10^6 cells/ml) were incubated for 60 min at 37° with 0.02% (v/v) diethylmaleate (DEM) as described previously [19]. After this period the cells were sedimented by centrifugation at 600 *g* for 4 min and viability determined by Trypan Blue exclusion before exposure to the quinone drugs. Control cells contained 8.7 ± 0.6 nmol GSH/10^6 cells (N = 6) compared with 2.7 ± 0.2 nmol/10^6 cells (N = 6) in DEM-treated cells. DEM treatment did not affect the viability of the initial cell suspensions (control cells were 86.3 ± 2.6% (N = 6) and DEM-treated cells were 82.5 ± 2.2% (N = 6) viable).

Inhibition of enzyme activities by quinones. Enzyme inhibition experiments were carried out in human adult liver microsomal and cytosolic fractions prepared from liver samples obtained at kidney donation as described previously [20]. Menadione or mitozantrone at concentrations up to 50 μM were added to microsomal incubations for measurement of EH and MFO activities. Epoxide hydrolase was measured using 20 μM phenanthrene-9,10-oxide and enzyme activity quantified by direct fluorimetric detection of the phenanthrene-9,10-dihydrodiol formed [21]. Cytochrome P-450 dependent mixed function oxidase was measured as the *O*-deethylation of ethoxyresorufin as described previously [13]. The concentrations of menadione and mitozantrone which could be tested were limited by quenching of the fluorescence of both phenanthrene-9,10-dihydrodiol and resorufin. The effects of menadione and mitozantrone on the activities of cytosolic glutathione-*S*-transferase (GST) and glutathione reductase and on microsomal NADPH–cytochrome *c* reductase were also determined. GST activity towards 50 μM 1-chloro-2,4-dinitrobenzene (CDNB) was measured in the presence of 1 mM GSH as described by Habig and Jakoby [22]. Glutathione reductase activity was measured by following the oxidation of NADPH as decribed previously [23] abd NADPH–cytochrome *c* reductase activity by the reduction of cytochrome *c* [24].

Interaction of quinones with GSH. Menadione (10–500 μM) and mitozantrone (10–500 μM) were incubated with 100 μM GSH for 30 min at 37° in Krebs–Henseleit buffer, pH 7.4, containing 10 mM HEPES

and the GSH remaining after this incubation was determined fluorimetrically [14].

RESULTS

Cytotoxicity studies

Menadione caused a concentration dependent decrease in cell viability (Fig. 1A) which was preceded by rapid depletion (within 5–10 min) of intracellular GSH content (Fig. 1B). Although concentrations of mitozantrone up to 200 μM had little effect on cell viability or GSH content afer 4 hr incubation, 500 μM caused a decrease in cell viability within 120 min (Fig. 2). This effect was not preceded by GSH depletion; the time course of GSH loss in mitozantrone-treated cells followed that of cell death (results not shown). For further experiments on the effect of enzyme inhibitors and GSH depletion on the toxicity of the two quinones, 10 μM menadione and 100 μM mitozantrone were used.

In the presence of dicoumarol, an inhibitor of DT-diaphorase activity, the toxicity of both quinones was exacerbated (Fig. 3A and B). As observed with the 500 μM mitozantrone alone, the cell death in the presence of dicoumarol and 100 μM mitozantrone was not preceded by depletion of GSH. Fig. 4 shows that inhibition of catalase by 1,2-aminotriazole increased the toxicity of menadione between 120 and 240 min of incubation, but did not affect the viability of cells incubated with mitozantrone. Inhibition of glutathione reductase by BCNU caused a dramatic increase in the toxicity of menadione, but had no effect on that of mitozantrone (Fig. 5). The inhibition of EH activity by TCPO did not affect the response of the cells to 10 μM menadione, but exacerbated the toxicity of 100 μM mitozantrone (Fig. 6). Prior depletion of intracellular GSH by pretreatment of the cells with DEM resulted in a marked increase in menadione toxicity (Fig. 7A) and to a smaller extent in mitozantrone toxicity (Fig. 7B). DEM pretreatment did not affect cell viability *per se*. The effect of the various enzyme inhibitors and of GSH depletion on the toxicity of the two quinones is summarised on Table 1.

Enzyme inhibition

The activities of microsomal EH, cytosolic GST and glutathione reductase in subcellular fractions of human adult liver were not affected by either menadione or mitozantrone at concentrations up to 100 μM. Microsomal NADPH–cytochrome *c* reductase activity was slightly inhibited (20%) by 50 μM menadione or mitozantrone. Both quinones inhibited MFO activity as measured by the *O*-deethylation of ethoxyresorufin. Figure 8 shows that menadione was a more potent inhibitor of the human liver MFO activity than was mitozantrone. Similar results were also observed with both quinones on the activity of pentoxyresorufin *O*-dealkylase (results not shown).

Incubations with GSH

The interaction of menadione (50–500 μM) and mitozantrone (10–500 μM) with GSH is demonstrated in Fig. 9. Menadione reacted with GSH in a concentration dependent manner, whereas mitozantrone, at the concentrations used here did not interact directly with GSH.

DISCUSSION

This study indicates that in the human liver-

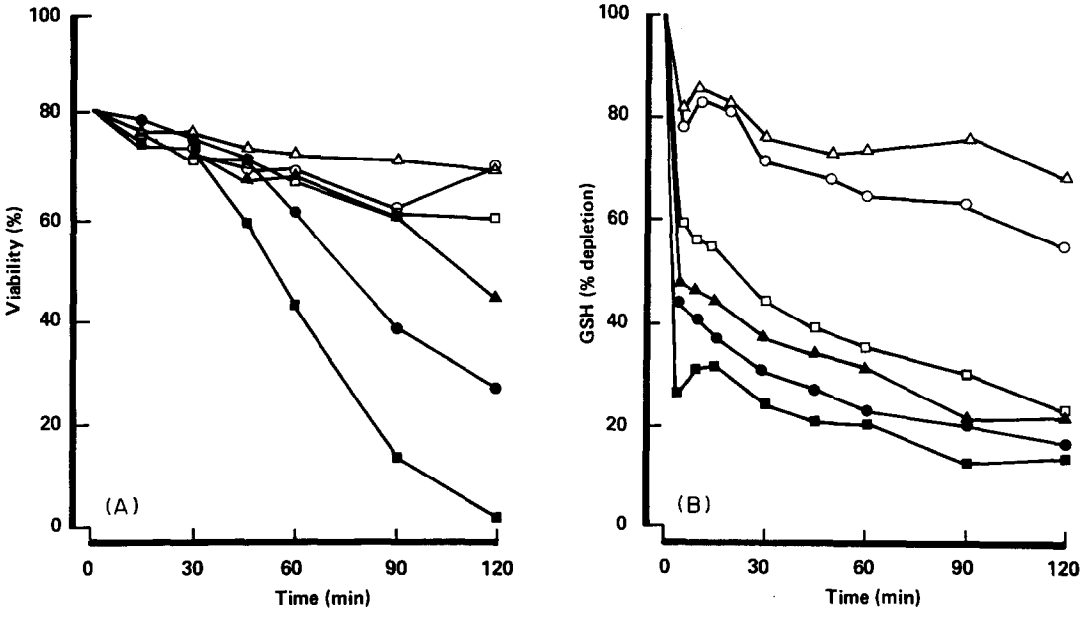

Fig. 1. Cell viability (A) and GSH depletion (B) in Hep G2 cells incubated in the absence of menadione (△, N = 5), and in the presence of 10 μM (○, N = 5); 25 μM (□, N = 5); 50 μM (▲, N = 5); 100 μM (●, N = 5) and 200 μM (■, N = 3) menadione. Results are presented as means, variation between experiments was less than 12%.

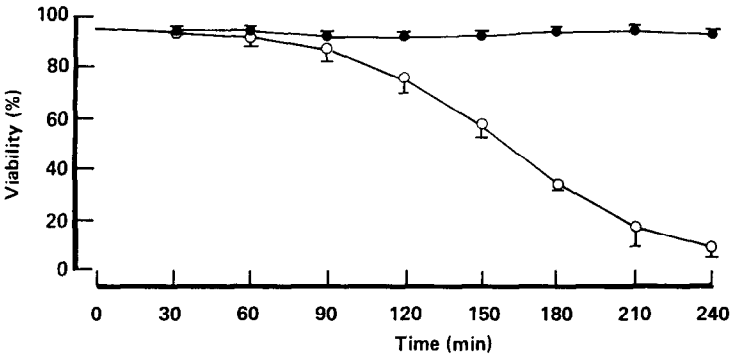

Fig. 2. The viability of cells incubated with 500 μM mitozantrone. ●, control incubations (N = 4) and ○, incubations containing 500 μM mitozantrone (N = 3). Error bars represent the SEM.

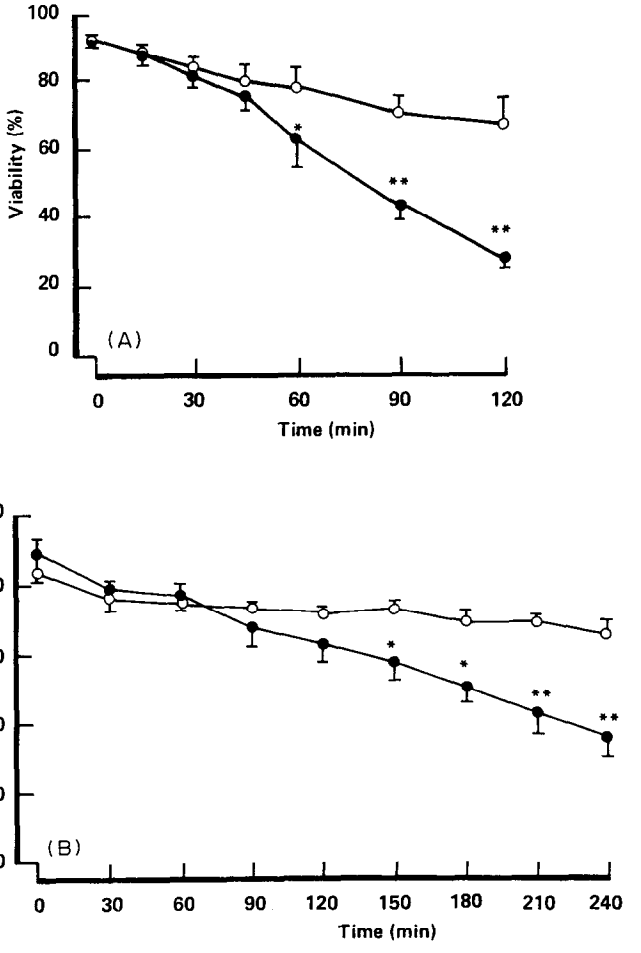

Fig. 3. The effect of dicoumarol on the viability of cells incubated with 10 μM menadione (A) or 100 μM mitozantrone (B). Open circles are results from cells incubated in the absence of dicoumarol, closed circles in the presence. Results are means ± SEM of 4 experiments for menadione and 11 for mitozantrone. * $P < 0.05$, ** $P < 0.001$, by unpaired Student's t-test. Significance values refer to differences between incubations in the presence and absence of dicoumarol.

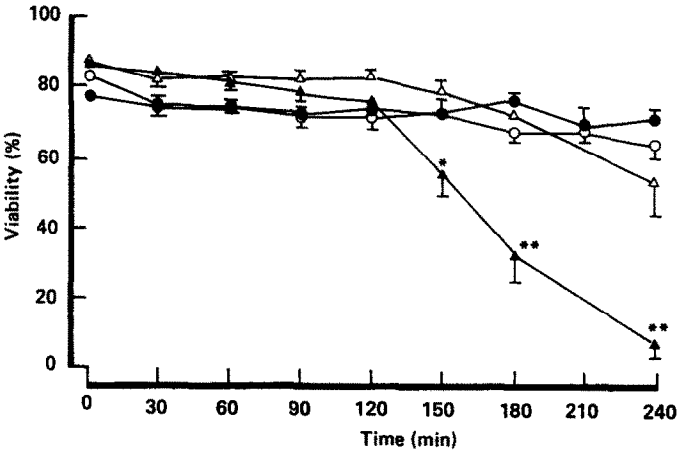

Fig. 4. The effect of 1,2-aminotriazole on the viability of cells exposed to 100 μM mitozantrone or 10 μM menadione. ○, 100 μM mitozantrone control cells (N = 7); ●, 100 μM mitozantrone in the presence of aminotriazole (N = 2); △, 10 μM menadione in control cells (N = 5); ▲, 10 μM menadione in the presence of aminotriazole (N = 5). Error bars are SEM where N > 2 and range where N = 2. * $P < 0.05$, ** $P < 0.005$, by unpaired Student's *t*-test. Significance values refer to differences between incubations with menadione in the presence and absence of aminotriazole.

derived Hep G2 cells mitozantrone cytotoxicity is not mediated through the one-electron reduction/oxidative stress mechanism which is involved in the cytotoxicity of quinone drugs such as menadione and adriamycin® [25, 26]. In support of this, inhibition of the enzymes glutathione reductase and catalase, responsible for protecting the cells from oxidative damage, did not affect the response of the Hep G2 cells to mitozantrone, whereas it exacerbated menadione toxicity. In addition, the toxicity of menadione was preceded by depletion of GSH. This has previously been shown to be due primarily to oxidation to the dimer GSSG by the products of oxidative stress generated by the one-electron reduction of the quinone [27]. In contrast, mitozantrone did not cause depletion of GSH prior to cell death.

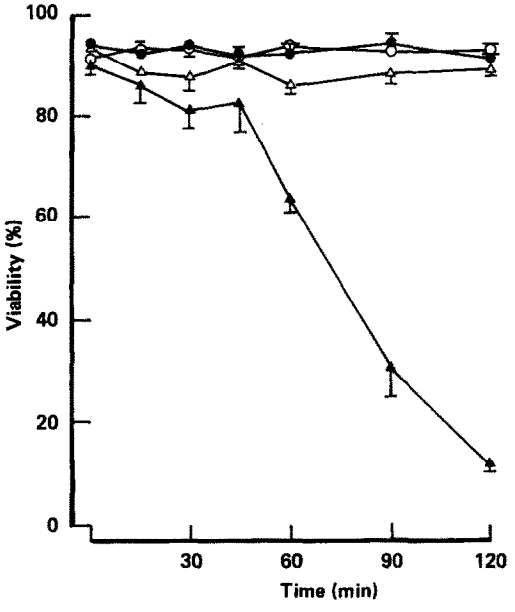

Fig. 5. The effect of BCNU on the viability of cells exposed to 100 μM mitozantrone or 10 μM menadione. ○, 100 μM mitozantrone control cells (N = 2); ●, 100 μM mitozantrone in the presence of BCNU (N = 2); △, 10 μM menadione in control cells (N = 2); ▲, 10 μM menadione in the presence on BCNU (N = 2). Error bars are the range.

Previous studies have shown that mitozantrone is a poor substrate for one-electron reduction by NADPH–cytochrome *c* reductase in rabbit liver compared with adriamycin® and menadione [26]. Moreover, mitozantrone does not significantly increase NADPH oxidation or superoxide formation in rat or rabbit liver microsomes [26, 28]. However, Basra and coworkers have demonstrated the generation of free radicals with both mitozantrone and adriamycin® in human liver microsomes supplemented with NADPH [29]. In these experiments adriamycin® also stimulated the rate of microsomal NADPH oxidation and superoxide formation in human liver whereas mitozantrone had little effect. These observations in human liver microsomes and the results of our experiments on Hep G2 cells suggest that the cytotoxic properties of mitozantrone may be mediated through a free radical mechanism other than drug-stimulated oxygen radical production and oxidative damage.

Our experiments suggest that mitozantrone toxicity may involve MFO activation to an epoxide intermediate. Mitozantrone has previously been shown to undergo oxidative metabolism catalysed by cytochrome P-450 [11] and horseradish peroxidase [30]. If the biological activation of mitozantrone is mediated through the MFO system, this, together with the low rate of reaction with NADPH–cytochrome *c* reductase, may explain why cardiac tissue is relatively resistant to mitozantrone toxicity. Heart tissue contains only a very low cytochrome P-450 content, whereas the activity of cardiac NADPH–

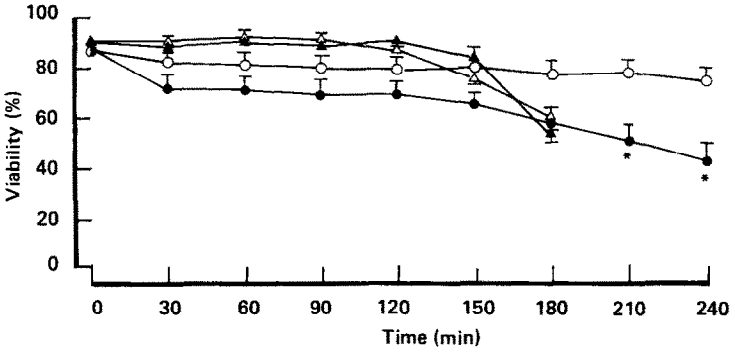

Fig. 6. The effect of TCPO on the viability of cells exposed to 100 μM mitozantrone or 10 μM menadione. ○, 100 μM mitozantrone in control cells (N = 7); ●, 100 μM mitozantrone in the presence of TCPO (N = 6); △, 10 μM menadione in control cells (N = 5); ▲, 10 μM menadione in the presence of TCPO (N = 6). Error bars are SEM. * $P < 0.05$, by unpaired Student's t-test. Significance values refer to differences between incubations with mitozantrone in the presence and absence of TCPO.

cytochrome c reductase supports the reduction of adriamycin efficiently [31]. In addition it may be of therapeutic value to administer an inducing agent, such as phenobarbitone, which increases MFO activities, concomitantly with mitozantrone therapy. The activation of mitozantrone to toxic metabolites by the MFO system is currently being investigated further.

Inhibition of DT-diaphorase by dicoumarol increased the toxicity of both menadione and mitozantrone, indicating that the two quinones were metabolised by this enzyme and that DT-diaphorase activity provides a protective mechanism in the cells. Hydroquinones generated by this reaction are readily conjugated with glucuronic acid and excreted from the cell.

Depletion of intracellular GSH by DEM caused a large increase in menadione toxicity, and had a lesser effect on that of mitozantrone. The oxidative stress induced by menadione metabolism in rat hepatocytes results in depletion of both soluble and protein thiols, and the latter effect is critically associated with loss of cell viability [26]. Loss of protein thiols occurs more rapidly in cells containing decreased levels of GSH, and this may explain the marked increase in menadione toxicity observed in DEM-treated Hep G2 cells. Mitozantrone toxicity in Hep G2 cells did not involve oxidation and depletion of GSH. In

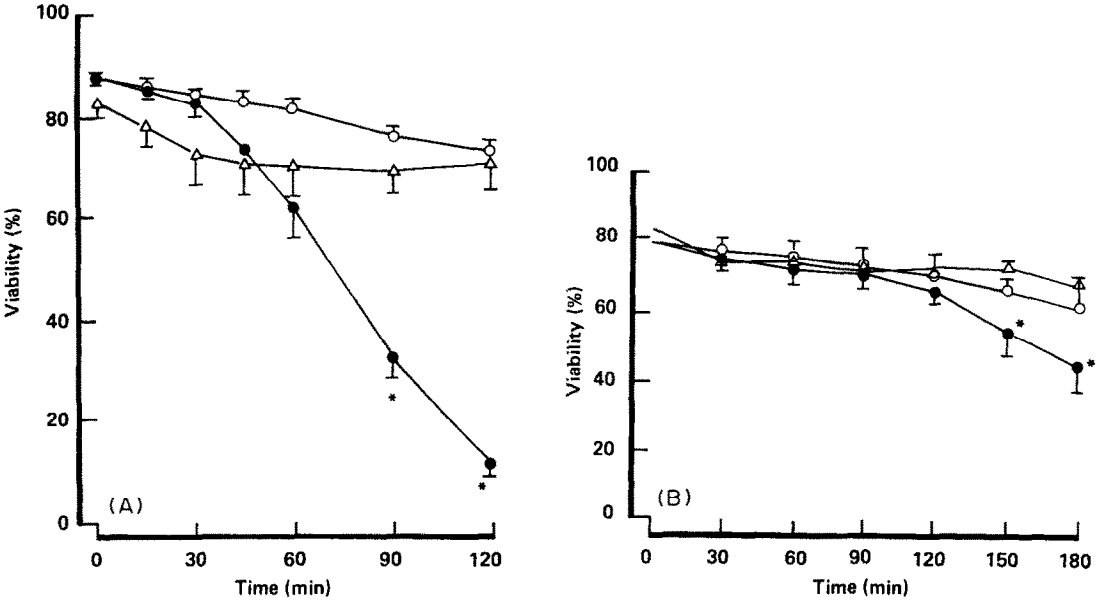

Fig. 7. The effect of diethylmaleate (DEM) pretreatment on the viability of cells incubated with 10 μM menadione (A) or 100 μM mitozantrone (B). ○ control cells with drug, △ DEM-treated cells without drug, ● DEM-treated cells with drug. Results are means ± SEM, where N = 4 for menadione and N = 6 for mitozantrone. * $P < 0.05$, by unpaired Student's t-test. Significance values refer to differences between incubations with the individual drugs in control cells and in DEM treated cells.

Table 1. The effect of enzyme inhibition and depletion of glutathione on the viability of cells exposed to menadione or mitozantrone

Incubations	120 min	10 μM Menadione [180 min] Cell viability (%)	240 min	100 μM Mitozantrone 240 min
Control	82.5 ± 1.9 (15)	—	84.5 ± 1.4 (7)	84.5 ± 1.4 (7)
Drug alone	77.2 ± 2.9 (15)	[59.3 ± 18.3] (5)	54.6 ± 9.2* (5)	65.3 ± 3.8* (4)
Drug + dicoumarol	27.8 ± 2.4† (4)	—	—	36.7 ± 5.1† (11)
Drug + aminotriazole	77.8 ± 2.8 (5)	—	8.8 ± 4.3‡ (5)	72.0, 75.5 (2)
Drug + BCNU	11.5, 11.0 (2)	—	—	90.0, 93.0 (2)
Drug + TCPO	—	[52.7 ± 8.9] (6)	—	48.8 ± 7.3 (6)
Drug + DEM	10.0 ± 3.2† (4)	—	—	22.9 ± 5.1† (6)

Results are means, with the number of experiments given in parentheses. Where N > 2, the SEM is shown and where N = 2 the individual values are given.

* $P < 0.05$, by one way analysis of variance followed by Dunnett's test. Significance values refer to differences between control incubations and those containing menadione or mitozantrone at 240 min.

† $P < 0.05$, by one way analysis of variance followed by Dunnett's test. Significance values refer to differences between menadione or mitozantrone alone and menadione or mitozantrone plus inhibitor or DEM treatment.

‡ $P < 0.05$, by non-paired Student's *t*-test. Significance value refers to difference between menadione alone and menadione plus aminotriazole at 240 min.

addition, we have shown that mitozantrone did not react directly with GSH whereas menadione interacted rapidly in a concentration-dependent manner. However, mitozantrone has been shown to be conjugated with GSH in rat liver microsomes and the formation of the GSH conjugate required prior activation of the quinone by MFO [11]. In the case of mitozantrone, depletion of intracellular GSH in the Hep G2 cells may increase toxicity by preventing the conjugation of reactive metabolite(s) generated by the MFO system. Depletion of GSH has little effect on the cytotoxicity, mutagenicity and DNA damage produced by adriamycin® in cultured mammalian cell lines [32], although it does exacerbate adriamycin® toxicity in rat hepatocytes [17].

Both quinones inhibited MFO activity in human liver microsomes: menadione was a more potent

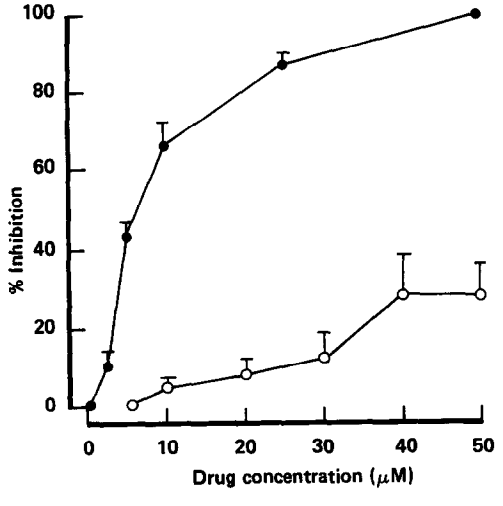

Fig. 8. Inhibition of ethoxyresorufin O-dealkylation by menadione (●, N = 3) and mitozantrone (○, N = 3) in human adult liver microsomal fractions. Error bars are SEM.

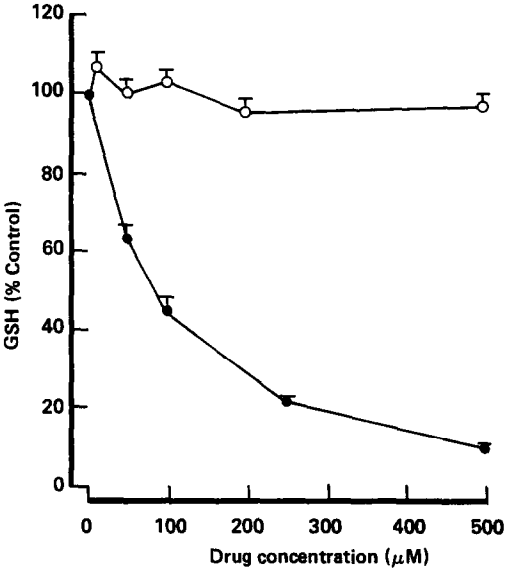

Fig. 9. Interaction of menadione (●, N = 3) and mitozantrone (○, N = 3) with GSH. Results are shown as % of the GSH remaining after 30 min incubation with the quinones. Error bars are SEM.

inhibitor than mitozantrone. The ability of quinones to inhibit MFO activity has been reported previously. Gillette and coworkers first showed that menadione inhibited antipyrene *N*-demethylation [33] and benzo(*a*)pyrene quinones are known to inhibit the oxidation of benzo(*a*)pyrene and its metabolites [34]. These inhibitory effects are proposed to occur due to an electron shunt which diverts reducing equivalents away from cytochrome P-450 towards NADPH–cytochrome *c* reductase and molecular oxygen. Since mitozantrone has been shown to be a poor substrate for NADPH–cytochrome *c* reductase, this may account for the less potent inhibitory effect this drug has on the oxidation of ethoxyresorufin. 1,4-bis(2-(2-Hydroxyethyl)amino)-ethylamino-9,10-anthracenedione, a quinone drug structurally similar to mitozantrone, inhibits MFO by preventing the function of NADPH–cytochrome *c* reductase [35]. However, neither menadione nor mitozantrone inhibited the activity of NADPH–cytochrome *c* reductase significantly and so this is unlikely to account for inhibition of the MFO system by these quinones.

The inhibition of MFO activities by quinone anticancer drugs requires further investigation as it may have important consequences that could affect their therapeutic use. Several anticancer drugs (for example cyclophosphamide) require activation by MFO to produce the active cytotoxic species. Since antineoplastic agents are often used in combination, the use of quinone drugs which inhibit MFO may result in decreased therapeutic effect.

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